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Stoichiometry of reconstituted high density lipoproteins in the hydrated state determined by photon antibunching

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ABSTRACT Apo A-I plays a central role in the in the structure of high density lipoproteins. Determining the stoichiometry of lipid-free and lipid-bound apo A-I in the hydrated state is therefore fundamental to understanding how HDL forms and function. Here we used the quantum optical phenomenom of photon antibunching to show that lipid-free Apo A-I consists of a mixture of monomers and dimers, whereas lipid-bound apo A-I forms a dimer, which is in agreement with the commonly accepted “double-belt” model.

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Apolipoprotein A-I (apo A-I) is the major protein found in high density lipoproteins (HDL) that plays a central role in the particle’s the structure and metabolism. HDL (a.k.a. “good cholesterol”) protects against atherosclerosis by carrying out reverse cholesterol transport, which delivers cholesterol from the tissues to liver for excretion, and also through its anti-inflammatory properties(1,2). HDL can be reconstituted in vitro with high reproducibility using spontaneous reaction of apo A-I with lipids. Reconstituted HDL (rHDL) is a discoidal complex approximately 10-20nm in size, which is also commonly referred to as a lipid nanodisc. Since the lipid structure of rHDL consists of a lamellar bilayer, they essentially provide a platform that mimics a nanoscopic, isolated patch of cell membrane (supported by the apoproteins). Such constructs have recently been used to reconstitute a variety of membrane proteins in a soluble colloidal particle, including cytochrome P450 and bacteriorhodopsin(3,4). These properties provide improved sample dispersion, characterization and homogeneity, which facilitate the biophysical and biochemical examination of isolated, functional membrane proteins(5,6). rHDL may also be a suitable alternative to liposomes as targeted drug transport vehicles(7,8). Due to the highly dynamic nature of the apoprotein-lipid interaction, many basic properties, e.g. their specific hydrated shape in solution, or the precise conformation of the apoproteins surrounding the particles are still uncertain. However, according to multiple lines of evidence, it is widely assumed that a single rHDL particle contains two molecules of apoprotein A-I (apo A-I) that circumscribe the perimeter of a disk-shaped lipid bilayer in a belt conformation(9-13). Here, we apply a quantum optical property of single fluorescent molecules to test the hypothesis that apo A-I exists as a dimer on rHDL in the hydrated state, which is difficult to determine with and might be masked by bulk averaging analysis techniques.

The quantum optical phenomenon of photon antibunching represents the ultimate manifestation of the quantized nature of light(14). It was first observed in the late 1970’s from resonance fluorescence of sodium atoms in atomic beams(15). Shortly afterwards, photon antibunching was shown in single fluorescent dyes trapped in a solid at low temperature(16). Due to advances in laser technology and detectors, it is now possible to observe antibunching at room temperature from fluorescent dyes(17-19). Recently, it has been shown that this phenomenon can also be an extremely powerful technique to count independent emitters in biomolecules. Fore et al. demonstrated this by using antibunching to distinguish between singly, doubly, and triply fluorescently labeled DNA oligonucleotides, while Sykora et al. applied this to study protein conformation in ion channels(20,21). Here, we apply photon antibunching to determine the stoichiometry of lipid-free and lipid-bound apolipoprotein A-I (apo A-I), and count their numbers in high density lipoproteins (HDL).

Photon antibunching exploits the fact that a single fluorescent molecule can only emit one photon at a time. In a simplified two level energy diagram, a molecule in an excited state requires a finite amount of time before it relaxes back to the ground state, with the temporal separation between adjacent photons being determined mostly by this excited state lifetime. Only two or more molecules can emit photons simultaneously. Photon antibunching is usually performed in a Hanbury-Brown and Twiss interferometer setup which equally splits the photons with a 50/50 beamsplitter and sends them to two detectors attached to a coincidence counter(22). Photons detected are then cross-correlated between the two channels in the

ps/ns regime, in which antibunching effects occur. By measuring the probability of detecting photon pairs as a function of time, $n_{pair}(t)$, one would find that for emission from a single quantum system, $n_{pair}(0) = 0$ (e.g. a single

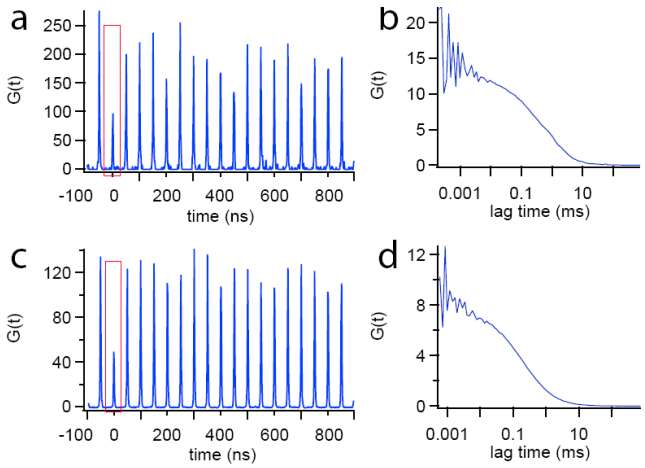

Figure 1. Photon antibunching histograms and fluorescence correlation spectroscopy of lipidbound (top) and lipid-free apoA-I (bottom). The central (antibunched) peak is located at time 0 and highlighted by a red box. A) Lipid-bound antibunching histogram and their corresponding FCS autocorrelation. B) Lipid-free antibunching histogram and their corresponding FCS autocorrelation.

molecule cannot emit two photons at the same time), but $n_{pair}(t)$ increases for time intervals greater than zero. Experimentally, this is equivalent to the measurement of the second-order autocorrelation function $G^{(2)}(t)$ defined as

$$G^{(2)}(t) = \frac{\langle I(t_0)I(t_0+t)\rangle}{\langle I(t)\rangle^2}$$

Using pulsed laser excitation, Tinnefeld et al demonstrated that photon antibunching can be used to count the number of fluorescent molecules *N*, via the formula $\frac{M_C}{\overline{M_l}} = 1 - \frac{1}{N}$ by measuring the area of a central photon count peak M_C, representing zero coincidence time, with respect to the area of all other lateral peaks $\overline{M_l}$ (19) in a photon coincidence diagram. The ratio $M_C / \overline{M_l}$ is a measure of the probability of detecting photon pairs per laser pulse. The central peak represents coincident photon pairs at time zero, and the lateral peaks are photon pairs correlated with time intervals of 50 ns, set equal to the laser repetition rate. In our experiment, the central peak is initially offset by 100 ns, which is a result of a BNC cable delay between the START-STOP channels of the coincidence counter, to prevent photon loss due to the inherent dead time of the electronics. This offset was compensated for in the histograms in Fig. 1. To account for background contributions all measurements were repeated with unlabled protein, resulting in a background signal of 3% of the fluorescent signal. Because of its minor contribution we did not correct for this background.

rHDL are synthesized using apo A-I and lipid vesicles with a 100:1 DMPC/apoA-I molar ratio. Prior to reconstitution, a clone of apo A-I is expressed containing a single cysteine using site-specific mutagenesis(23). This apo A-I clone is then reacted with Alexa488-maleimide derivatives via sulfhydril-maleimide chemistry and subsequent purification in a separation column to result in fluorescently-labled apo A-I where each apo A-I carries just a single fluorophore. For accurate stoichiometry measurements, we further purified the rHDL complex using HPLC to eliminate unbound proteins and lipids, and to select a fraction that corresponds to 150 kDa which represents the typical molecular weight of rHDL.

We conduct our experiments with a MicroTime 200 confocal fluorescence microscope system (PicoQuant, Berlin) equipped with a 470 nm pulsed diode laser (80 ps pulsewidth, 20 Mhz repetition rate) and time correlated single photon counting (TCSPC) electronics (Picoharp 300). The laser is focused through an Olympus 1.45 NA 100x oil objective to a diffraction-limited spot of 250 nm diameter at a height of 5µm above the glass coverslip surface. The emission is split by 50/50 beamsplitter and directed to two avalanche photodiode detectors (SPCM-AQR-14, PerkinElmer).

To ensure single molecule statistics, the concentration of lipid-free and lipid-bound apo A-I is diluted to below 1 nanomolar. The G(0) value of the auotocorrelation obtained from monitoring freely diffusing rHDL in PBS by laser excitation using fluorescence correlation spectroscopy (FCS) accounts to greater than 7 which leads to a particle number in the detection volume of less than 1/7=0.15. This low number indicates a high probability that only one particle is present in the detection volume at any given point in time, which is a prerequisite for antibunching analysis. Using FCS, we measured an average diffusion time of 600 ± 25 µs over multiple measurements which corresponds to a diffusion coefficient of 26±1 $\mu m^2/s$ (Fig 1A, right). The hydrodynamic radius of the rHDL is calculated to be 9.3 ± 0.4nm via the Stokes-Einstein equation $D = KT/(6\pi n R_H)$, but it should be kept in mind that the shape of these particles is likely not spherical as assumed for this model.

For antibunching analysis, we conduct 5 measurements of 15 minutes each for both the lipid-bound and lipid-free apoA-I in PBS. We determined a value of $M_C / \overline{M_l} \approx 0.49$ which gives N=1.96 ± 0.12 for rHDL, in excellent agreement with the value of two apoA-I assuming that two proteins are bound to the lipids in the commonly accepted double-belt conformation (Fig 2A). In contrast, for lipid-

free apo A-I, FCS provides a diffusion rate of approximately 80 $\mu m^2/s$. Here, $M_C / \overline{M_l} \approx 0.41$ which gives N=1.67 ± 0.10, leading us to conclude that lipid-free apo A-I can form a heterogeneous population consisting mostly of monomers and dimers. However since photon antibunching is based on photon statistics, this does not rule out the formation of higher order oligomers, which may occur depending on the concentration of the sample(24,25). Thus, we have shown that by applying the quantum optical technique of photon antibunching, we are able to determine structural parameters of lipid-free and lipid-bound rHDL in their hydrated state that are difficult, if not impossible to obtain by other means. This method will prove highly valuable for future investigation into the stoichiometry of spherical HDL, of which, although representing a predominant physiological form, far less is understood about their structure and protein stiochiometry.

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